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Award Number: DAMD17-01-1-0183

TITLE: Functional Analysis of LIM Domain Proteins and Co-Factors in Breast Cancer

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REPORT DATE: October 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2004	Annual Summary (17 Sep 2001 - 16 Sep 2004)

4. TITLE AND SUBTITLE
Functional Analysis of LIM Domain Proteins and Co-Factors in Breast Cancer

5. FUNDING NUMBERS
DAMD17-01-1-0183

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

We identified a novel transcription factor, LMO4, which is highly expressed in breast epithelial cells during mid-pregnancy when these cells are proliferating and invading the stroma. Since previous members of the LIM only (LMO) gene family are oncogenes, we hypothesized that LMO4 may play roles in mammary gland development and cancer. We have now shown that expression of LMO4 correlates with proliferation, and in transgenic mice we showed that dominant-negative LMO4 inhibits lobuloalveolar development, demonstrating that LMO4 plays roles in proliferation and/or invasion of breast epithelial cells. Because these cellular features are associated with breast carcinogenesis and because LMO4 is overexpressed in a subset of breast cancers, our studies implicate LMO4 as a possible oncogene in breast cancer. In addition, we found that the LMO4 gene is activated by the Her2/Neu receptor in breast cancer cells, providing further linkage to breast cancer. In biochemical assays we showed that LMO4 may act by associating with the GATA3 transcription factor, also expressed in mammary epithelial cells. We have also created stable breast cancer cell lines in which we can induce expression of LMO4 and Clim2. With this method, we have identified several target genes of LMO4, one of which is Bone Morphogenic Protein 7 (BMP-7), which can affect survival of breast cancer cells by regulating apoptosis. In summary, we have defined a role for a new gene, LMO4, in mammary epithelial cell proliferation in normal development and in breast cancer cells.

14. SUBJECT TERMS
Cellular differentiation, cellular proliferation, transcription factors, transgenic mice, protein-protein interactions

15. NUMBER OF PAGES
29

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

LIM only factors, LMOs, are nuclear proteins composed of two LIM domains and little other sequence. Two of the founding members of this family are act as oncogenes in lymphocytes. We have recently identified a new member of this family, LMO4, which is highly expressed in proliferating epithelial cells, including those of the breast. In addition, LMO-4 has been shown to be highly expressed in ductal breast carcinoma (1). Two mechanisms of action have been proposed for LMOs. First, they may bind the LIM homeodomain co-activator CLIM thereby interfering with the activity of LIM homeodomain proteins. Second, LMOs may associate with DNA-binding proteins, thus attracting the coactivator CLIM to DNA. Our hypothesis is that LMO4 plays a role in regulation of breast epithelial cell proliferation and that subversion of its function may play a role in breast cancer. Further, we propose that LMO4 acts, at least in part, by associating with DNA-binding proteins.

Our specific aims were: #1. To test the potential role of the LMO4/CLIM complex in breast development and breast cancer by using the MMTV promoter to overexpress LMO4 and a dominant negative form of CLIM in breast epithelial cells. #2. We identified a KRAB Zinc finger transcription factor, Znf43, as a potential interacting partner of LMO4. We proposed to test the relevance of this interaction using human breast carcinoma cell lines. We proposed to test the effect on proliferation and tumor growth.

BODY

Task 1. **Test the effect of overexpressing LMO-4 and a dominant negative CLIM in the breast of transgenic mice.** Tasks 1-1 to 1-5 are technical in nature and are described in our paper (Oncogene 2004:23, 1507-1513; (2)), which is included with the Appendix.

1. Create and characterize transgenic constructs.

The initial plan was to create two lines of transgenic mice: MMTV-LMO4 and MMTV-dominant negative-Clim. In addition, I have worked on a third transgenic line where we have created a dominant negative LMO4 molecule by linking LMO4 to the Engrailed repression domain. This construct is referred to as MMTV-Engrailed-LMO4. All three transgenic constructs have been created and characterized.

2. Injecting transgenic constructs into oocytes.

This was accomplished early in the project, see previous Progress Report and (2).

3. Analyses of founder mice.

This was accomplished early in the project, see previous Progress Report and (2).

4. Generating transgenic progeny.

This was accomplished early in the project, see previous Progress Report and (2).

5. Analyses of transgenic mice.

To test the effect of the Engrailed-LMO4 molecule on mammary gland development, we placed it under control of the MMTV promoter (Fig. 3 in (2)), which directs high expression in epithelial cells of mammary glands in transgenic mice and has been extensively used for this purpose (3-6). The fusion protein was HA tagged to allow its immunodetection in mammary glands. Of five transgenic lines, three independent lines expressed the transgene in mammary gland epithelial cells. Expression of the transgene was found both in virgin and pregnant mammary glands (Fig. 3 in (2)) and was predominantly nuclear (Fig. 3 in (2)).

To evaluate the effects of expressing the Engrailed-LMO4 fusion protein in mammary gland epithelial cells, we examined mammary gland development by whole mount analyses in transgenic mice and compared them to wild-type littermates. Development of transgenic mammary glands of virgin mice was normal at 3 to 4 weeks (data not shown), but at 6 weeks a mild delay in the progression of ductal development was evident (Fig. 3 in (2)). At 8 weeks, most transgenic mammary glands were normal (Fig. 3 in (2)), although we did observe occasional abnormality at that stage. These data indicate that the Engrailed-LMO4 fusion protein causes a transient delay in mammary gland development of virgin mice. In pregnant transgenic mice, a clear delay in alveolar development was evident at day 5.5 (Fig. 4 in (2)); this delay, however, was later overcome and by day 15.5, lobuloalveolar development was essentially normal (Fig. 4 in (2)). No abnormalities were observed during lactation (Fig. 4 in (2)) and transgenic females were able to nurse normal-size litters. In conclusion, expression of the dominant negative Engrailed-LMO4 fusion protein in the mammary glands of mice results in the slowing of ductal development in virgin mice and a transient inhibition of alveolar development during pregnancy. These results are consistent with our hypothesis derived from the expression analyses and indicate that LMO4 is likely to play roles to promote invasion and/or proliferation of mammary gland epithelial cells.

We have not observed a clear phenotype in the MMTV-dominant negative-Clim lines. Analyses of the MMTV-LMO4 lines showed that this transgene was expressed at very low level, precluding analyses of these mice. To troubleshoot this, we have generated MCF-7 breast cancer cell lines in which we can induce expression of LMO4. These cell lines will allow us to investigate the effect of LMO4 overexpression in breast cancer.

To test the effect of LMO4 in mammary epithelial cells, we have profiled expression in MCF-7 cells expressing LMO4 in a conditional manner (Fig.1, top panel). For these experiments, we selected 3 independent cell clones and profiled expression under basal conditions (control conditions, in the presence of doxycycline) and under induced conditions (LMO4 expression, 7 days after doxycycline withdrawal). To decrease variability, RNA samples from two independent experiments were pooled for each of the three cell clones. We hybridized to U133A and B Affymetrix chips, which contain 44,692 probe sets, and analyzed the data with the Cyber-T program, which was developed at UCI (7). This statistical data package, which is especially suitable for pairwise comparisons, uses a Bayesian statistical framework to determine the local confidence (p-values) based on the *t*-test distribution of individual gene measurements. Thus, for each experimental condition, we can obtain: a mean expression level, a fold-change between control and experimental condition, and a *t*-value to establish the

confidence level of the observed difference in expression of a particular gene between control and experimental conditions.

Overview of the data processing is provided in Fig. 1 (lower panel). Using probability criteria of $p \leq 0.05$, 888 probe sets (805 genes) were altered after LMO4 induction. Of these 805 genes, 431 are upregulated and 374 are downregulated. Interestingly, this experiment suggests that not only can LMO4 stimulate gene expression, but also repress a group of genes. Table 1 shows a list of the top genes (listed in order of increasing p value) showing differential expression after induction of LMO4 in MCF-7 cells. Many of the target genes, which we have independently validated with quantitative PCR, are involved in oncogenesis. In an effort to further understand the role of LMO4, we subjected the significantly altered genes to pathway analyses (Ingenuity Systems). The results show (Table 2) that the pathways significantly affected by LMO4 are those involved in cellular proliferation and apoptosis. Therefore, the microarray data from breast cancer cells is consistent with our mouse developmental data: LMO4 plays roles in mammary epithelial cell survival, most likely by affecting cell proliferation.

In summary, in human breast cancer cells, LMO4 alters the expression of several genes involved in oncogenesis.

Task 2. **Test the relevance of LMO4/Znf43 interactions in breast cancer cell lines.**

1. Creating constructs and cell lines transfections.

We initiated characterization of LMO4 interacting factors by creating LMO4 and Clim2 expressing vectors that can be induced by tetracycline (Tet-on system). However, expression in these cell lines was not stable and therefore, we have switched to the Tet-off system. The LMO4 and the dominant-negative Clim (DN-Clim) proteins are tagged with Myc and the Clim2 protein is tagged with HA, thus allowing specific immunoprecipitation of these proteins from breast cancer cell lines. The construction of both vectors was completed and several cell lines were generated (Figs. 1 and 2).

2. Performing in vitro interaction assays in vitro and in cells.

The LMO2 oncogene, which is highly related to LMO4 is known to interact with GATA factors (8). This suggested the possibility that LMO4 might also interact with GATA factors. Of GATA factors, GATA3 has been shown to be expressed in breast cancer cells (9). We therefore evaluated its expression during mammary gland development in the mouse and demonstrated that GATA3 is expressed throughout mammary gland development, and especially highly during pregnancy. In addition, we tested whether LMO4 is capable of interacting with GATA3, using co-immunoprecipitations of extracts from the MCF-7 cells stably expressing Myc tagged LMO4. In this assay, we were able to demonstrate an *in vivo* interaction between GATA3 and LMO4. In summary, LMO4 may act by associating with GATA3 in normal and malignant mammary epithelial cells.

3. Creating stable cell lines for analyses.

Because LMO4 and Clim are thought to act as a complex, we tested the transcriptional effect of DN-Clim, using the inducible cell line (Fig. 2, top panel).

Microarray analysis was performed as described above for the LMO4 cell lines. We found that 579 probe sets (524 genes) were altered by DN-Clim; 337 and 187 genes were upregulated and downregulated, respectively (Fig. 2, lower panel). As predicted, there is significant overlap in the genes regulated by LMO4 and DN-Clim (Fig. 3). However, unexpectedly, the genes that were altered both by LMO4 and DN-Clim expression were all regulated in the same direction, but not in opposite direction as the current models suggest.

Among the target genes upregulated by both LMO4 and DN-Clim were BMP-7 and IGFBP5, which has been implicated in breast cancer. We therefore cloned the promoters of these genes to study the mechanism of transcriptional regulation by LMO4. In transient transfection assays, both LMO4 and DN-Clim upregulated the BMP-7 and IGFBP5 promoters, suggesting that the regulation by LMO4/Clim is direct on these genes (Fig. 4 and 5). Consistent with this finding, LMO4 was found to associate with the BMP-7 and IGFBP5 promoters in chromatin immunprecipitation assays (Fig. 6). These findings have allowed us to create new models for how LMO4 may act on a transcriptional level in breast cancer cells (Fig. 7). Based on our work, we propose that LMO4 disrupts Clim-containing complexes; when these complexes contain repressors, the disruption results in activation of gene expression and when these complexes contain co-activators, the disruption results in repression of gene expression.

KEY RESEARCH ACCOMPLISHMENTS

1. Definition of LMO4 and Clim2 gene expression during mammary gland development.
2. Showing that LMO4 is a target gene of Her2/Neu.
3. Generating transgenic mice expressing LMO4, Clim2 and DN-Clim under the MMTV promoter.
4. Showing that MMTV-Engrailed-LMO4 mice exhibit defective ductular development in virgin mice and defective alveolar development in pregnant mice.
5. Identifying several target genes of LMO4 using microarray analyses, many of which are involved in oncogenesis.
6. Demonstrating expression of GATA3 in mammary glands of mice.
7. Demonstrating an in vivo interaction between LMO4 and GATA3.
8. Creation of stable breast cancer cell lines expressing tagged LMO4, DN-Clim and Clim2 proteins, using the Tet-off system.
9. Defining the genes altered by the expression of DN-Clim in breast cancer cells.
10. Defining the transcriptional mechanism by LMO4 in breast cancer cells.

REPORTABLE OUTCOMES TO DATE

1. Transgenic mouse models for LMO expression
2. Permanent breast cancer cell lines expressing tagged LMO4, DN-Clim and Clim2 in an inducible manner

3. Manuscript: **Wang, N.**, Kudryavtseva, E., Chen, I., Sugihara, T.M., McCormick, J., and Andersen, B. 2003. Expression of an Engrailed-LMO4 fusion protein in mammary epithelial cells inhibits mammary gland development in mice. *Oncogene,* 2004: 23, 1507-1513.
4. Abstract: **Wang, N.**, Kudryavstseva, E., Chen, I., Sugihara, T., & Andersen, B. 2002. The potential role of a new LIM factor, LMO4, in breast cancer. Proceedings Era of Hope Meeting, Orlando Florida, September (Abstract P4-1).
5. Abstract: **Wang, N.**, & Andersen, B. 2003. The potential role of a new LIM factor, LMO4, in breast cancer. California Breast Cancer Research Program meeting in San Diego, California, September.
6. Wang N, Kudryavtseva E, Chen I, McCormick J, Sugihara TM, Ruiz R, **Andersen B.** Heregulin/HER2 regulation of LMO4 in breast cancer cells. *Endocrine Society Annual Meeting,* New Orleans, June 16 – 19, 2004 (Abstract P2-281).

CONCLUSIONS

In summary, with the support of the Army Fellowship Award, I have been able to acquire expertise in working on the breast cancer problem at the level of molecular genetics. At the same time, I have defined the role for a new transcription factor, LMO4, in mammary gland development and in breast cancer. I have already published one paper in *Oncogene* and three abstracts as a first-author paper describing some of my findings. In addition, I am preparing a manuscript for submission that describes my most recent findings with LMO4 in breast cancer.

During the funding period, I made significant progress on both specific aims, and my training in breast cancer biology has been greatly enhanced. Our results show that LMO-4 expression is associated with undifferentiated breast epithelial cells such as those found during mid-pregnancy and in breast cancer. The major achievements are (1) the finding that interfering with LMO4 in breast epithelial cells leads to inhibition of ductular and alveolar development in mice, (2) the identification of several LMO4 target genes, many of which are involved in oncogenesis, (3) the identification of GATA3 as an LMO4-interacting transcription factor, and (4) the demonstration that LMO4 acts in breast cancer cells to disrupt transcriptional complexes containing the co-factor Clim2. Our findings strengthen the hypothesis that overexpression of LMO4 may contribute to breast carcinogenesis.

With our work, we hope to generate new ideas about treatment of breast cancer, thus impacting on reducing the human/economic cost of breast cancer. I sincerely thank the Army for their support, and I am convinced that my work will play a role in solving the breast cancer problem.

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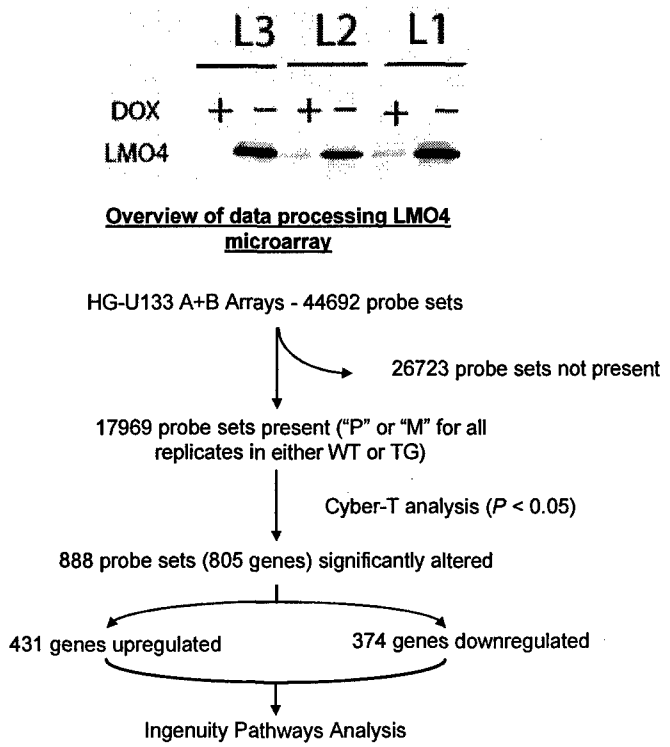

Figure 1. Microarray analysis of gene expression induced by LMO4 in tet-off MCF7 cell lines. Top panel shows that LMO4 is expressed in conditional manner in three different cell lines (L1, L2 and L3), and lower panel shows overview of data processing of the LMO4 microarray results.

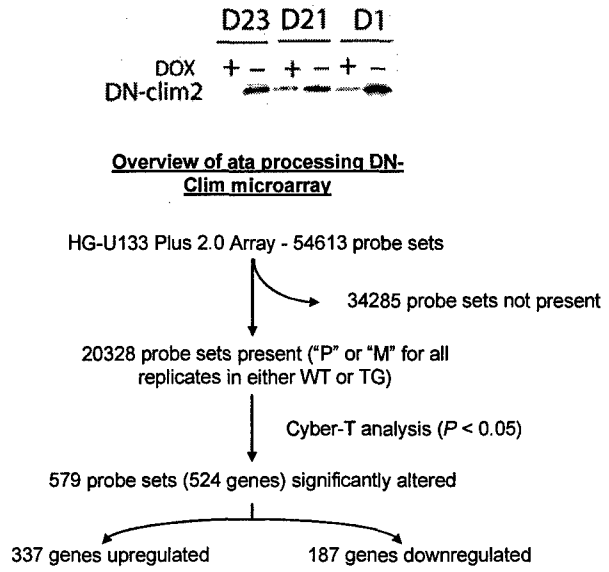

Figure 2. Microarray analysis of gene expression induced by DN-Clim2 in tet-off MCF7 cell lines. Top panel shows that DN-Clim2 is expressed in a conditional manner in three different cell lines (D1, D21 and D23), and lower panel shows overview of data processing of the DN-Clim2 microarray results.

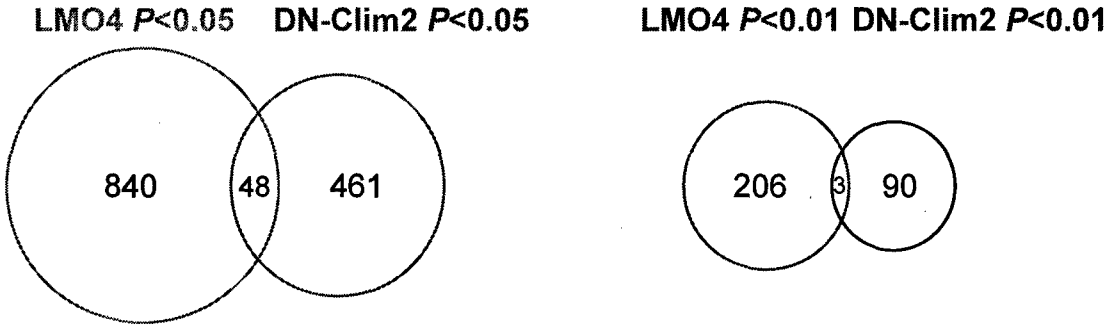

Figure 3. Overlap between genes regulated by LMO4 and DN-Clim2 in our microarray datasets.

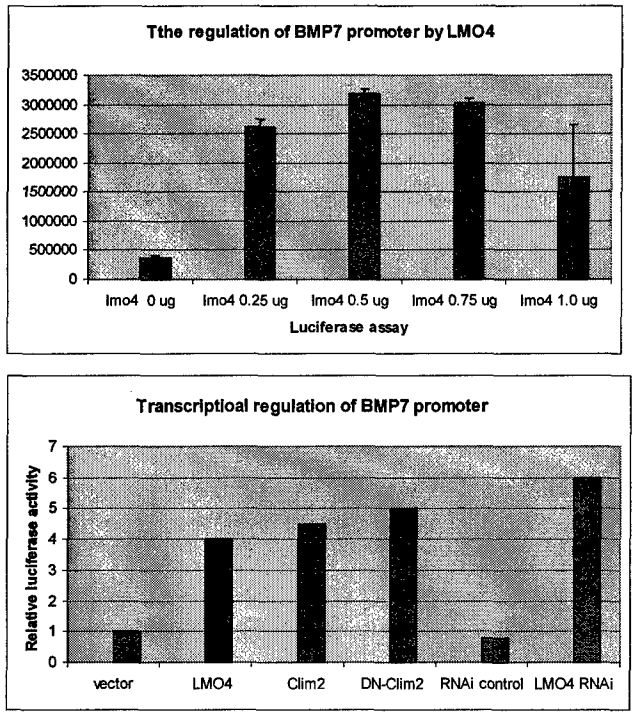

Figure 4. Transcriptional regulation of the BMP7 promoter. Top panel shows that LMO4 can increase BMP7 promoter activity in dose dependent fashion; lower panel shows that LMO4, Clim2, DN-Clim2, and LMO4 RNAi all can increase the BMP7 promoter activity.

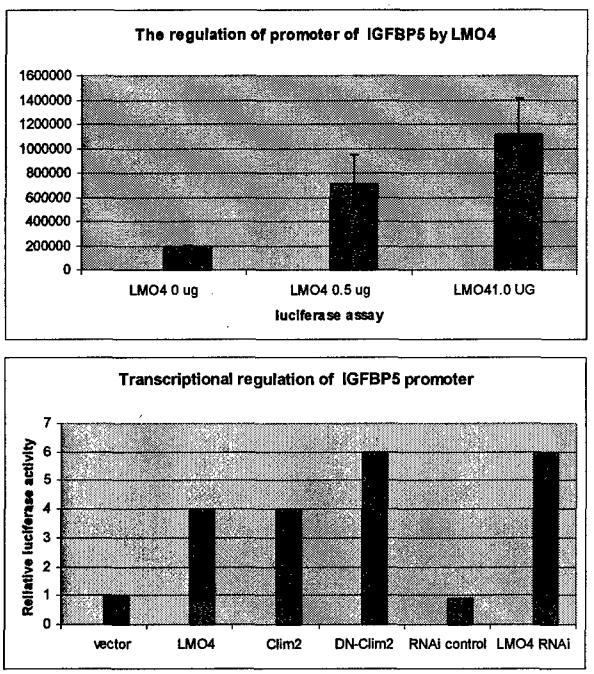

Figure 5. Transcriptional regulation of the IGFBP5 promoter. Top panel shows that LMO4 can increase IGFBP5 promoter activity in dose dependent fashion. Low panel shows that LMO4, Clim2, DN-Clim2, and LMO4 RNAi can increase the IGFBP5 promoter activity.

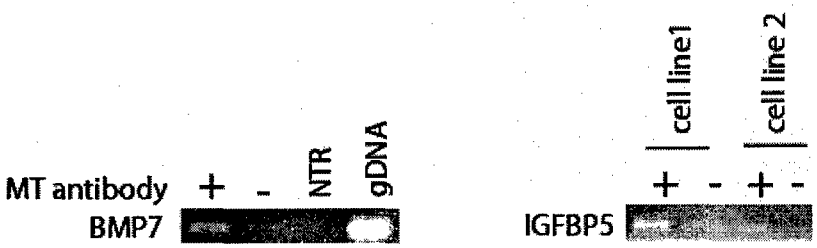

Figure 6. Chromatin imunoprecipitation assays. Cellular chromatin of inducible LMO4 MCF7 cell lines was crosslinked with formaldehyde after LMO4 induction, protein – DNA complexes were incubated with myc antibody and isolated by immunoprecipitation. PCR was performed for the amplification of BMP7 and IGFBP5 promoters. The left panel shows that LMO4 can bind to the BMP7 promoter, and the right panel shows that LMO4 can bind to the IGFBP5 promoter. Negative controls were isolated by immunoprecipitation without antibody (-), NTR: non-template control, g DNA: genomic DNA.

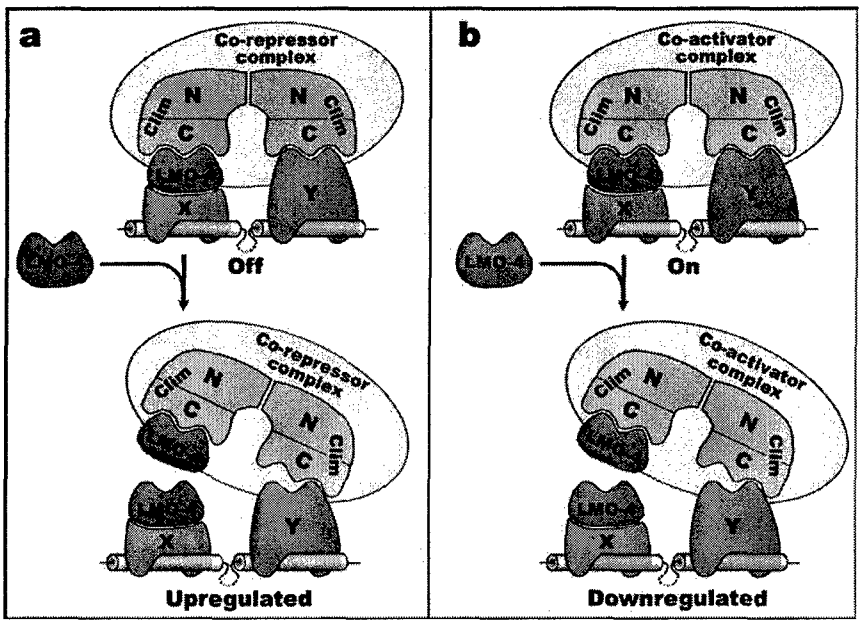

Figure 7. A new model for how LMO4 acts at a transcriptional level in breast cancer cells. LMO4 disrupts Clim-containing complexes. When these complexes contain repressors, the disruption leads to activation of gene expression, and when these complexes contain co-activators, the disruption results in repression of gene expression

Probe set ID	Accession	Gene symbol	Gene name	*P* value	Fold
201369_s_at	NM_006887	ZFP36L2	zinc finger protein 36, C3H type-like 2	2.86E-05	1.82
212593_s_at	N92498	PDCD4	programmed cell death 4	1.14E-04	2.00
215771_x_at	X15786	RET	ret proto-oncogene	1.23E-04	1.91
202428_x_at	NM_020548	DBI	diazepam binding inhibitor	1.62E-04	1.57
204326_x_at	NM_002450	MT1X	metallothionein 1X	2.06E-04	1.73
211259_s_at	BC004248	BMP7	bone morphogenetic protein 7	2.50E-04	2.21
201334_s_at	AB002380	ARHGEF12	Rho guanine nucleotide exchange factor (GEF) 12	2.58E-04	1.76
224671_at	AL571373	MRPL10	mitochondrial ribosomal protein L10	1.01E-03	1.58
202948_at	NM_000877	IL1R1	interleukin 1 receptor, type I	1.11E-03	1.86
204573_at	NM_021151	CROT	carnitine O-octanoyltransferase	1.22E-03	1.97
226722_at	BE874872	FAM20C	family with sequence similarity 20, member C	1.27E-03	1.68
225433_at	AU144104	GTF2A1	general transcription factor IIA, 1, 19/37kDa	1.31E-03	1.50
40093_at	X83425	LU	Lutheran blood group (Auberger b antigen included)	1.55E-03	1.56
204112_s_at	NM_006895	HNMT	histamine N-methyltransferase	1.78E-03	1.66
200028_s_at	NM_020151	STARD7	START domain containing 7	1.92E-03	1.48
217009_at	AL121974	PGK2	phosphoglycerate kinase 2	2.00E-03	2.36
201242_s_at	BC000006	ATP1B1	ATPase, Na+/K+ transporting, beta 1 polypeptide	2.40E-03	1.60
209240_at	AF070560	OGT	O-linked N-acetylglucosamine (GlcNAc) transferase	2.53E-03	1.47
223000_s_at	AF172398	F11R	F11 receptor	2.54E-03	1.35
212525_s_at	AA760862	H2AFX	H2A histone family, member X	2.63E-03	1.63

Probe set ID	Accession	Gene symbol	Gene name	*P* value	Fold
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231713_s_at	NM_018255	STATIP1	signal transducer and activator of transcription 3 interacting protein 1	2.21E-04	-1.47
233208_x_at	AA583986	CPSF2	cleavage and polyadenylation specific factor 2, 100kDa	2.36E-04	-1.68
229558_at	AI927643	MGC16824	esophageal cancer associated protein	3.80E-04	-1.81
233588_x_at	BE561798	HKE2	HLA class II region expressed gene KE2	6.99E-04	-1.91
238346_s_at	AW973003	NCOA6IP	nuclear receptor coactivator 6 interacting protein	8.11E-04	-1.82
225415_at	AA577672	BBAP	rhysin 2	8.40E-04	-1.42
238496_at	AA741074	WHSC1L1	Wolf-Hirschhorn syndrome candidate 1-like 1	1.14E-03	-2.51
212444_at	AA156240	RAI3	retinoic acid induced 3	1.16E-03	-1.51
225496_s_at	N21426	SYTL2	synaptotagmin-like 2	1.33E-03	-1.34
218462_at	NM_025065	RPF1	RNA processing factor 1	1.47E-03	-1.64
207626_s_at	NM_003046	SLC7A2	solute carrier family 7, member 2	1.52E-03	-1.60
220319_s_at	NM_013262	MYLIP	myosin regulatory light chain interacting protein	1.71E-03	-1.51
236300_at	BF698797	PDE3A	phosphodiesterase 3A, cGMP-inhibited	1.78E-03	-1.74
225152_at	BF940944	ZNF622	zinc finger protein 622	2.45E-03	-1.39
208430_s_at	NM_001390	DTNA	dystrobrevin, alpha	2.70E-03	-1.60
223211_at	BC001627	HPCL2	2-hydroxyphytanoyl-CoA lyase	2.86E-03	-1.42
205774_at	NM_000505	F12	coagulation factor XII (Hageman factor)	2.92E-03	-1.52
232337_at	AK000770	B3GNT7	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 7	3.16E-03	-1.54
240027_at	BF062244	LIN7A	lin-7 homolog A (C. elegans)	3.16E-03	-2.14

Table 1. List of the top genes upregulated and downregulated by LMO4 in MCF7 cells. Top panel represents upregulated genes by LMO4 and lower panel represents downregulated genes by LMO4.

Function	Network Genes	*P* value
proliferation	AKT1, CEBPA, CTNNB1, ETS2, **GADD45A**, **GATA2**, NR2C2, RET, TIAM1	8.29E-04
	CD47, CXCR4, DMTF1, IL1R1, ITGB5, MAX	4.90E-05
	BMP7, E2F3, IGFBP5, **ITCH**, **OCLN**, TCF3	2.88E-03
	GRN, SKP2	2.85E-02
	IGFBP2, MTA1	2.85E-02
apoptosis	AKT1, BCL6, **CDC42**, CEBPA, CTNNB1, ETS2, **GADD45A**, HNRPA1, **PAK2**, RET, TCF7L1, TIAM1	9.40E-06
	CD47, CXCR4, MAX, **ODC1**	2.46E-03
	CFLAR, **TNFRSF18**	1.42E-05
	ACVR1B, BMP7, **SMAC**, TCF3	9.01E-03
invasion	AKT1, **CDC42**, CTNNB1, ETS2, TIAM1	2.37E-03
adhesion	CTNNB1, TIAM1	1.49E-02
migration	CD47, CXCR4, ITGB5, MAX	8.33E-08
	CD151, **MDK**	2.70E-03
transformation	DMTF1, MAX, **ODC1**	2.95E-05

Table 2. Ingenuity Pathways Analysis of LMO4 microarray data. The results show that the pathways significantly affected by LMO4 are those involved in cell proliferation and apoptosis.

Oncogene (2004) 23, 1507–1513

www.nature.com/onc

Expression of an engrailed-LMO4 fusion protein in mammary epithelial cells inhibits mammary gland development in mice

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LIM domain factors and associated cofactors are important developmental regulators in pattern formation and organogenesis. In addition, overexpression of two LIM-only factors (LMOs) causes acute lymphocytic leukemia. The more recently discovered LMO factor LMO4 is highly expressed in proliferating epithelial cells, and frequently overexpressed in breast carcinoma. Here we show that while LMO4 is expressed throughout mammary gland development, it is dramatically upregulated in mammary epithelial cells during midpregnancy. The LMO coactivator Clim2/Ldb1/NLI showed a similar expression pattern, consistent with the idea that LMO4 and Clim2 act as a complex in mammary epithelial cells. In MCF-7 cells, LMO4 transcripts were upregulated by heregulin, an activator of ErbB receptors that are known to be important in mammary gland development and breast cancer. To test the hypothesis that LMO4 plays roles in mammary gland development, we created an engrailed-LMO4 fusion protein. This fusion protein maintains the ability to interact with Clim2, but acts as a dominant repressor of both basal and activated transcription when recruited to a DNA-regulatory region. When the engrailed-LMO4 fusion protein was expressed under control of the MMTV promoter in transgenic mice, both ductular development in virgin mice and alveolar development in pregnant mice were inhibited. These results suggest that LMO4 plays a role in promoting mammary gland development.
Oncogene (2004) **23**, 1507–1513. doi:10.1038/sj.onc.1207288
Published online 15 December 2003

Keywords: LMO4; mammary gland development; heregulin; engrailed fusion protein

Introduction

The LIM motif, a cysteine-rich zinc-coordinating domain that mediates protein–protein interactions, was originally discovered as a component of homeodomain transcription factors (reviewed in Bach, 2000). A second class of LIM domain transcription factors, composed almost entirely of two tandem LIM domains, is referred to as LIM-only (LMO) proteins. Two members, LMO1 and LMO2, are oncoproteins found at sites of chromosomal translocations in acute T-cell leukemia (Rabbitts *et al.*, 1999). LMO proteins do not bind DNA directly, but regulate gene transcription by associating with other transcription factors. This model is supported by studies showing that LMOs, through their LIM domains, exist in a stable complex with helix loop helix (HLH) heterodimeric partner proteins that include TAL1(SCL)/E12 (Valge-Archer *et al.*, 1994; Wadman *et al.*, 1994, 1997; Osada *et al.*, 1995, 1997; Larson *et al.*, 1996; Visvader *et al.*, 1997; Ono *et al.*, 1998; Bao *et al.*, 2000; Herblot *et al.*, 2000; Mead *et al.*, 2001), and GATA factors (Osada *et al.*, 1995; Wadman *et al.*, 1997; Ono *et al.*, 1998; Mead *et al.*, 2001). In addition, LIM domains of the LIM homeodomain and LMO proteins interact strongly with cofactors, including the coactivators Clim1 and Clim2/Ldb1/NLI (Agulnick *et al.*, 1996; Jurata *et al.*, 1996; Bach *et al.*, 1997, 1999; Visvader *et al.*, 1997), which confer transcriptional activation and promote synergism between DNA-binding proteins (Bach, 2000).

Based on the prominent expression of Clim2 in proliferating epithelial cells of the epidermis and hair follicles, we discovered LMO4 as a Clim2-interacting protein in the epidermis (Sugihara *et al.*, 1998). LMO4, simultaneously discovered by other laboratories (Grutz *et al.*, 1998; Kenny *et al.*, 1998), is the main LIM domain factor expressed in proliferating epithelial cells of the epidermis and hair follicles (Sugihara *et al.*, 1998). Interestingly, the human *LMO4* gene was initially cloned from a breast cancer cDNA library (Racevskis *et al.*, 1999), and subsequent studies showed it to be overexpressed in more than half of all invasive breast carcinomas (Visvader *et al.*, 2001). Furthermore, LMO4 and Clim2 overexpression interfered with differentiation of cultured mammary epithelial cells (Visvader *et al.*, 2001).

The goals of our studies were to establish a dominant-negative LMO4 molecule that can be used to repress transcription of LMO4 target genes and to study the

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Received 7 July 2003; revised 24 September 2003; accepted 13 October 2003

biological function of LMO4 in the mammary gland *in vivo*. We show that fusion of the repression domain from the *Drosophila* engrailed homeobox protein (Han and Manley, 1993) to LMO4 creates a strong transcriptional repressor, capable of interfering with basal and activated transcription. Expression of this fusion molecule under the MMTV promoter in mammary glands of transgenic mice leads to inhibition of ductular and alveolar development, suggesting that LMO4 is involved in progression of mammary gland development.

LMO4 is upregulated in mammary epithelial cells during midpregnancy and by heregulin in MCF-7 breast cancer cells

To gain insights into the role of LMO4 in mammary gland biology, we assessed its expression in mouse mammary gland and breast cancer cell lines. In contrast to a previous study employing Northern blot analyses on total RNA (Visvader *et al*., 2001), the sensitive RNAse protection assays show that LMO4 transcripts are easily detected in virgin mammary glands and that expression levels remain relatively stable from age 4 weeks to 14 weeks (Figure 1a). However, there is dramatic upregulation of LMO4 in mammary glands from midpregnancy, with levels falling late in pregnancy (Figure 1a), and a moderate increase in LMO4 levels during early lactation (Figure 1a). Clim2 levels are coordinately regulated during mammary gland development, with the highest levels found in midpregnancy (Figure 1a), consistent with the idea that LMO4 and Clim2 act as a complex. *In situ* hybridization studies on mammary gland sections show that LMO4 is primarily expressed in ductular and alveolar epithelial cells (Figure 1b). Consistent with the RNAse protection assay experiments, LMO4 levels are high at day 14.5 and lower at day 18.5 (Figure 1b). The surge in LMO4/Clim2 transcript levels during midpregnancy suggests an especially important function at this developmental stage, characterized by dramatic epithelial cell proliferation and stromal invasion.

In three different human breast cancer cell lines, LMO4 transcript levels vary from high in the estrogen receptor-negative MDA-MB-231, intermediate in the estrogen receptor-negative MDA-MB-453, to low in the estrogen receptor-positive MCF-7 (Figure 1c). Estradiol did not increase LMO4 expression in MCF-7 cells (Figure 1c), consistent with findings in human breast cancer indicating that LMO4 is especially characteristic for estrogen receptor-negative tumors (Gruvberger *et al*., 2001). In contrast to the coordinately regulated expression of LMO4 and Clim2 during normal mammary gland development (Figure 1a), Clim2 levels remain constant in breast cancer cell lines that express high levels of LMO4 transcripts (Figure 1c) and protein (Figure 1d). These results suggest that relative overexpression of LMO4 compared to Clim2 may be important for LMO4 actions in breast cancer. Since LMO4 may be localized to the cytoplasm under certain conditions (Kenny *et al*., 1998), we evaluated its cellular distribution in breast cancer cells by generating MCF-7 cells stably expressing an myc-tagged (MT) LMO4. In these cells, LMO4 is restricted to the nucleus (Figure 1e). In contrast to the lack of estrogen regulation, LMO4 expression is stimulated by the ErbB ligand heregulin, which is known to be important for alveolar maturation and proliferation (Figure 1f). Heregulin is thought to act through ErbB2-containing heterodimers (Stern, 2003) and its effect was partially blocked by an ErbB2 antibody (Figure 1g), suggesting a role for ErbB2 in heregulin-mediated upregulation of LMO4.

The observation that LMO4 may be downstream of heregulin/ErbB2 is consistent with findings that the mesenchymally expressed heregulin α, like LMO4, is strikingly upregulated in midpregnancy (Yang *et al*., 1995). In addition, heregulin and the ErbB2/ErbB3/ErbB4 receptors, which have growth-stimulatory roles (Krane and Leder, 1996; Aguilar *et al*., 1999), are particularly important for alveolar morphogenesis (Yang *et al*., 1995; Jones *et al*., 1996, 1999; Jones and Stern, 1999; Li *et al*., 2002). ErbB2 is also overexpressed in 15–40% of breast cancer cases, where it is associated with increased invasiveness and metastasis, as well as poor prognosis (Slamon *et al*., 1989; Eccles, 2001). Our findings suggest the possibility that LMO4 may participate in heregulin/ErbB signaling in the mammary gland.

An engrailed-LMO4 fusion protein is capable of protein–protein interactions and acts as a strong transcriptional repressor

LMO4 forms a complex with Clim coactivators in epithelial cells and is thought to be recruited to DNA-binding proteins, resulting in transcriptional activation of target genes. We hypothesized that fusing the *Drosophila* engrailed transcriptional repression domain to LMO4 would create a dominant-negative molecule capable of suppressing LMO4 target genes (Figure 2a). When fused to heterologous transcription factors, the engrailed repression domain confers strong transcriptional repression. This quality was successfully used to obtain insights into the biological function of a spectrum of transcriptionally active molecules, including c-Myb (Taylor *et al*., 1996), *Xenopus* tailless (Hollemann *et al*., 1998), GATA factors (Sykes *et al*., 1998; Dasen *et al*., 1999; Liu *et al*., 2002), homeobox factors iroquois3 (Kudoh and Dawid, 2001) and RaxL (Chen and Cepko, 2002), and β-catenin (Montross *et al*., 2000).

To test whether the engrailed-LMO4 fusion protein was capable of protein–protein interactions, we performed co-immunoprecipitation assays in HEK293T cells transfected with expression plasmids encoding tagged LMO4 and Clim2, as well as fusion proteins with LMO4 and Clim2. As expected, Clim2 antiserum precipitated LMO4 in cells transfected with Clim2 and LMO4 (Figure 2b). In cells cotransfected with tagged VP16-Clim2 and engrailed-LMO4 fusion proteins, both proteins could be precipitated independent of whether the precipitating antibody was directed against VP16-MT-

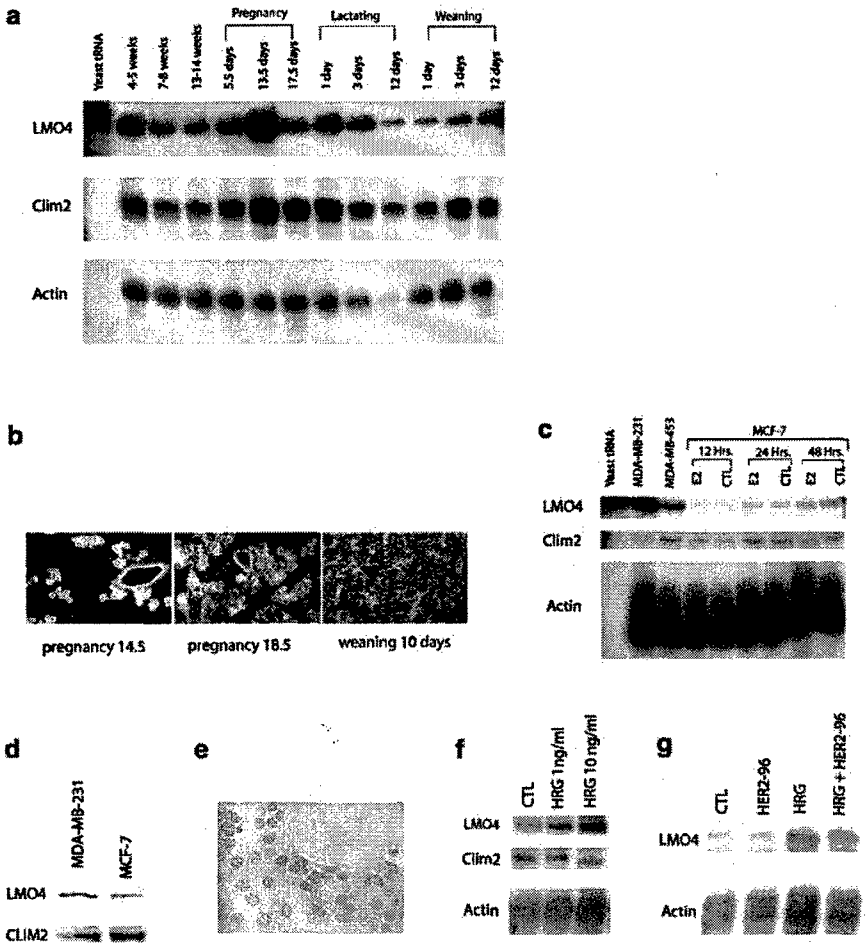


Figure 1 Expression of LMO4 and Clim2 during mammary gland development and in response to regulators of mammary gland development. **(a)** RNAse protection assays showing expression of LMO4 (top panel), Clim2 (middle panel), and β-actin (bottom panel) during the indicated stages of mammary gland development. **(b)** *In situ* hybridization study showing expression of LMO4 in mammary glands at day 14.5 of pregnancy (left panel), day 18.5 of pregnancy (middle panel), and day 10 after weaning (right panel). ^{35}S-labeled cRNA probes specific for mouse LMO4 were applied to formalin-fixed tissue, as described (Sugihara *et al.*, 1998). **(c)** RNAse protection assays showing expression of LMO4 (top panel), Clim2 (middle panel), and β-actin (lower panel) in the indicated breast cancer cell lines and with estradiol (E2) treatment (20 ng/ml) for the indicated times. MCF-7 cells were grown in the presence of phenol red-free media and charcoal-stripped serum. **(d)** Western blot of whole-cell extracts from MDA-MB-231 and MCF-7 cells, using rat LMO4 antibody (Sum *et al.*, 2002) and rabbit Clim antisera (Bach *et al.*, 1999). **(e)** Immunolocalization of LMO4 in MCF-7 cells stably expressing myc-tagged LMO4. After fixing with formalin, slides were incubated with a myc antibody and signal detected with peroxidase. **(f)** RNAse protection assays showing expression of LMO4 (top panel), Clim2 (middle panel), and β-actin (lower panel) in MCF-7 cells after heregulin treatment with the indicated concentrations for 24 h. MCF-7 cells were maintained in serum-free media. Similar effects were observed after 48 h treatment (data not shown). **(g)** RNAse protection assays showing expression of LMO4 (top panel) and β-actin (lower panel) in MCF-7 cells after treatment for 20 h with heregulin and ErbB2-blocking antibody (Clone Her2-96, Sigma). RNA isolation and RNAse protection assays were carried out as previously described (Andersen *et al.*, 1997), using ^{32}P-labeled cRNAs specific for mouse and human LMO4, Clim2, and β-actin

Clim (Figure 2c, left panel) or HA-engrailed-LMO4 (Figure 2c, right panel). We conclude that the fusion of the *Drosophila* engrailed repression domain to LMO4 does not interfere with its ability to interact with Clim proteins.

Since natural target genes for LMO4 are unknown, we tested the effectiveness of the engrailed-LMO4 fusion in a GAL reporter system, where we monitored the transcriptional activity of a luciferase reporter gene under the control of GAL DNA-binding sites and a minimal promoter (Sugihara *et al.*, 1998) (Figure 2d). While LMO4-GAL (Figure 2d, panel 2) has little effect on the basal activity of the promoter, engrailed-LMO4-GAL (Figure 2d, panel 3) represses transcription of the reporter gene 29-fold. Furthermore, engrailed-LMO4-GAL could completely overcome a 105-fold activation conferred by the recruitment of a Clim-VP16 fusion protein (Figure 2d, compare panels 5 and 6). Clim alone is a weak activator in this system and the Clim-VP16 fusion protein is used because the viral VP16 transactivation domain can confer strong transactivation to heterologous proteins. In summary, these experiments suggest that an engrailed-LMO4 fusion protein can repress both basal and activated expression of LMO4 target genes, and that this fusion molecule may be useful to test the biological functions of LMO4.

Expression of the engrailed-LMO4 fusion protein in mammary gland epithelial cells of mice interferes with mammary gland development

To test the effect of the engrailed-LMO4 molecule on mammary gland development, we placed it under control of the MMTV promoter (Figure 3a), which has been extensively used to direct a high expression in epithelial cells of mammary glands in transgenic mice (Muller *et al.*, 1988; Guy *et al.*, 1992; Kitsberg and Leder, 1996; Krane and Leder, 1996). Three independent lines expressed the transgene in mammary gland epithelial cells. Expression of the transgene was found both in virgin and pregnant mammary glands (Figure 3b), and by immunohistochemistry with an HA antibody expression was predominantly nuclear (Figure 3c). The relatively constant level of the transgene expression (Figure 3b) is probably because the transgene in this line is upregulated at the very end of pregnancy, as has been described for other MMTV transgenic mice (Jones *et al.*, 1999). We examined mammary gland development by whole mount analyses in transgenic mice and compared them to wild-type littermates. Development of transgenic mammary glands of virgin mice was normal at 3–4 weeks (data not shown), but at 6 weeks a mild delay in the progression of ductal development was evident (Figure 3d). At 8 weeks, most transgenic mammary glands were normal (Figure 3d, lower TG panel at 8 weeks), although we did observe occasional abnormality at that stage (Figure 3d, upper TG panel at 8 weeks).

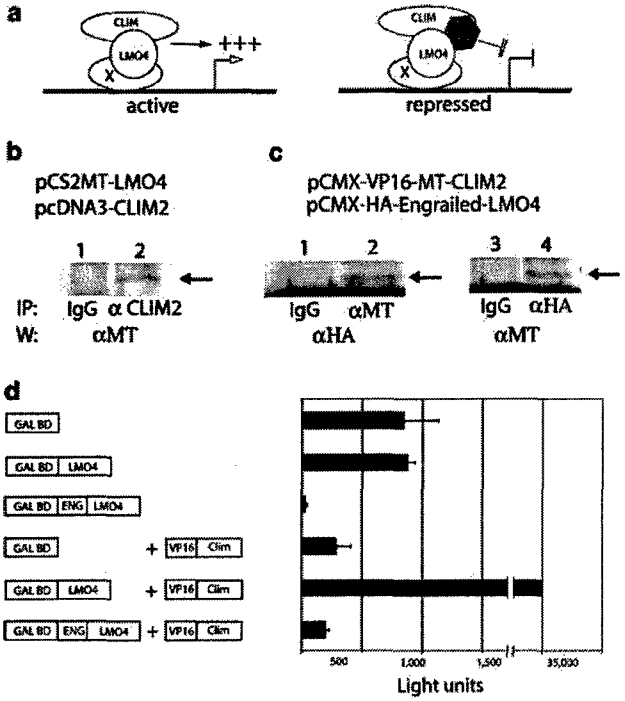
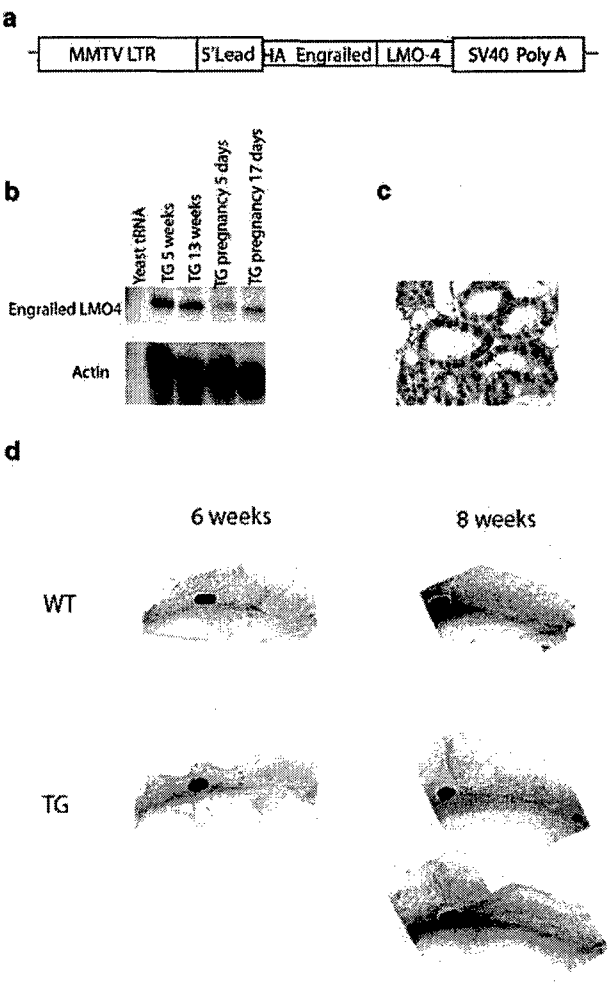


Figure 2 Interactions between Clim2 and LMO4 fusion proteins. (**a**) A model for the activity of the engrailed-LMO4 fusion protein. Under normal condition (left panel), LMO4/Clim complexes are thought to be recruited to promoters of target genes by associating with DNA-binding proteins (indicated as X), resulting in transactivation. Recruitment of the engrailed-LMO4 fusion proteins to the same complexes should result in transcriptional repression. (**b**) Immunoprecipitation of cell lysates from HEK293T cells transfected with expression plasmids encoding myc-tagged (MT) LMO4 and Clim2, using IgG (lane 1) and Clim antibody (Bach *et al.*, 1999) (lane 2). Western blot was probed with MT antibody (Invitrogen). (**c**) Immunoprecipitation of cell lysates from HEK293T cells (Sugihara *et al.*, 2001) transfected with expression plasmids encoding HA-tagged engrailed-LMO4 and myc-tagged VP16-Clim2, using IgG (lanes 1 and 3), MT antibody (lane 2), and HA antibody (lane 4). Western blots were probed with the indicated antibodies. The HA-engrailed-LMO4 fragment was generated in the mammalian expression vector pCMX by fusing the repression domain representing amino acids 2–299 of the Drosophila engrailed gene (Han and Manley, 1993) to a HA tag at the N-terminus and the full-length LMO4 coding sequence at the C-terminus. The pCMXGAL-LMO4 and pCMXGAL-engrailed-LMO4 plasmids contain the full-length LMO4 cDNA and the HA-engrailed-LMO4 fusion protein linked to the GAL DNA-binding domain. The pCMXVP16-Clim plasmid contains the C-terminal LIM-interaction domain of Clim1 (Bach *et al.*, 1999) linked to the VP16 transactivation domain. (**d**) The indicated GAL DNA-binding domain fusion proteins and VP16 fusion proteins were transfected into HEK293T cells with a GAL-luciferase reporter plasmid, using calcium-mediated gene transfer (Sugihara *et al.*, 1998). The results, expressed as light units, represent the mean and standard deviation from triplicate transfections. IP, immunoprecipitation; W, Western blot



These data indicate that the engrailed-LMO4 fusion protein causes a transient delay in mammary gland development of virgin mice.

In pregnant transgenic mice, a clear delay in alveolar development was evident at day 5.5 (Figure 4a and b); this delay, however, was later overcome, and by day 15.5 lobuloalveolar development was essentially normal (Figure 4b). No abnormalities were observed during lactation (Figure 4b) and transgenic females were able to nurse normal size litters. In conclusion, expression of the dominant-negative engrailed-LMO4 fusion protein in the mammary glands of mice results in the slowing of ductal development in virgin mice and a transient inhibition of alveolar development during pregnancy, suggesting that LMO4 plays roles in both ductular and alveolar development *in vivo*.

The phenotype of the MMTV-HA-engrailed-LMO4 mice may be distinct from the expected phenotype of LMO4 null mice. First, the engrailed-LMO4 fusion protein can suppress the expression of LMO4 target genes both under basal and activated conditions. In contrast, deletion of the LMO4 gene is likely to affect only the genes where LMO4 is actually participating in regulated transcription. Second, while it is generally thought that LMOs in combination with Clims are involved in transactivation, LMO4 may also participate in repression of certain genes, as has been suggested with BRCA1-mediated transcriptional activity (Sum *et al.*, 2002). The engrailed-LMO4 fusion protein would not be expected to affect these genes since they are already repressed. Finally, LMO4 may also act by binding to and sequestering other proteins in solution, a process the engrailed-LMO4 fusion protein would not be expected to inhibit. Such mechanisms have been proposed for the effect of *Drosophila* lmo in the fly wing (Zeng *et al.*, 1998).

The effect of engrailed-LMO4 expression in mammary glands was most clearly observed in early pregnancy, but the defect was overcome towards the end of pregnancy. Such defects, in which mammary gland development is slowed but not blocked, have been previously described in other genetically modified mice such as those with mutations in the ErbB2 gene (Stern, 2003). However, it is not possible to conclude that the role of LMO4 is restricted to ductular development in virgin mice and alveolar development in early pregnancy, because it is impossible to determine which levels of transgene expression are required to block endogenous LMO4 protein levels. Despite these limitations of the dominant-negative approach, our results strongly

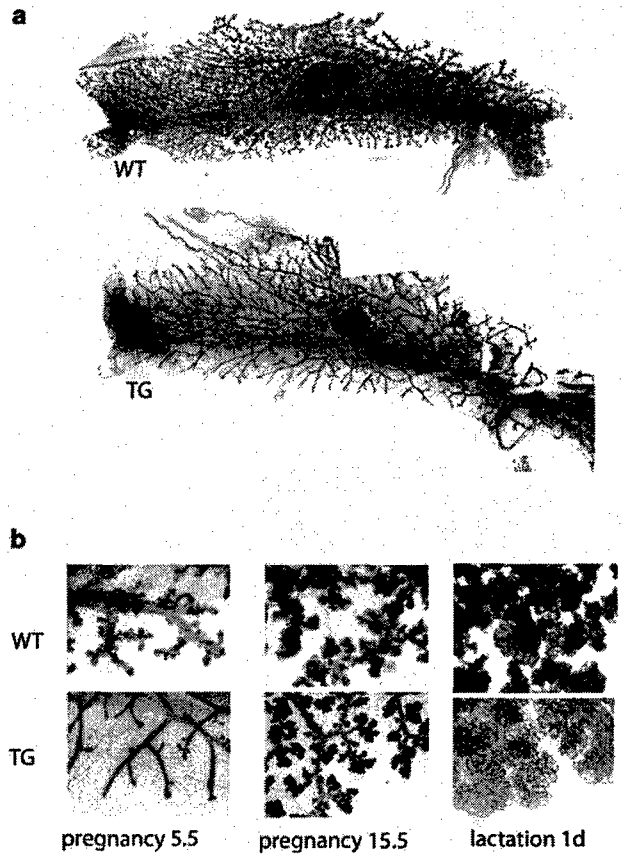


Figure 4 Effect of the engrailed-LMO4 fusion protein on mammary gland development during pregnancy. (**a**) Whole mount overview of mammary glands from 5.5-day pregnant mice comparing wild-type (WT) and transgenic (TG) mice. (**b**) Whole mount analyses in higher magnification from the indicated developmental stages. All magnifications are the same. Representative results from the analyses of 10 (5.5 day), six (15.5), and four (lactating) transgenic mice are shown

Figure 3 Effect of the engrailed-LMO4 fusion protein on mammary gland development in virgin mice. (**a**) A schematic of the transgene. The MMTV-HA-engrailed-LMO4 plasmid was created by cloning the HA-engrailed-LMO4 fragment into the *Eco*R1 site of the MMTV-SV40-BSSK plasmid (Leder *et al.*, 1986). To generate transgenic mice, the plasmid was cut with *Xho*1 and *Spe*1 to remove extraneous sequences, and the purified DNA fragment was then injected into fertilized CB6F1 oocytes, which were implanted into pseudopregnant mice. Of 13 mice born, five contained the MMTV-HA-engrailed-LMO4 sequences, as assessed by PCR with oligonucleotides specific for MMTV sequences. Of these five lines of founder mice, three (lines #1, 2, and 7) expressed the transgene, as assessed by immunohistochemistry with HA antibody on pregnant mammary glands. The three expressing lines were expanded by breeding into CB6F1 mice. Experiments were carried out with transgenic mice derived from lines #1, 2, and 7, which showed a comparable level of abnormality in mammary gland development. (**b**) RNAse protection assays showing expression of the engrailed-LMO4 transgene from line #7 at the indicated developmental time points. The probe, which corresponded to the *Drosophila* engrailed part of the fusion molecule, was specific because no signal was observed in mammary glands from wild-type mice (data not shown). (**c**) HA immunostaining of mammary gland (day 1 of lactation) from MMTV-HA-engrailed-LMO4 mice. Immunostaining of wild-type littermates gave no staining with the HA antibody (data not shown), indicating that the staining is specific. The mammary glands were fixed for 1 h at room temperature in a solution composed of six parts of ethanol, three parts of water, and one part of formaldehyde, followed by storage in 70% ethanol at 4°C. Paraffin-embedded tissue sections were stained with a monoclonal HA antibody (Covance) using peroxidase. (**d**) Whole mount staining of the fourth inguinal mammary glands from MMTV-HA-engrailed-LMO4 (TG) mice and littermate wild-type (WT) controls at the indicated developmental stages. Representative results from analyses of 16 (6 weeks) and three (8 weeks) TG mice are shown. The mammary glands were dissected, processed as a whole mount, fixed and stained with hematoxylin as described (Brisken *et al.*, 1999), and photographed at the same magnification

support roles for LMO4 in both ductular and alveolar development. Moreover, the dominant-negative LMO4 is a promising tool to evaluate the possible role of LMO4 in signaling pathways and in breast cancer.

The etiology of sporadic breast cancers is multifactorial and thought to involve stepwise mutations in several oncogenes and tumor-suppressor genes. The findings described in this paper are of importance because there are parallels between mammary epithelial cells during pregnancy and in breast cancer, and the LMO4 gene is frequently overexpressed in breast cancer. While neoplastic breast epithelial cells clearly have properties distinct from epithelial cells of the developing breast, the two also share similarities such as active proliferation and lack of terminal differentiation (Rudland *et al.*, 1998). Our studies – showing high expression of LMO4 during a stage in mammary gland development when there is active proliferation and stromal invasion, and the inhibition of these processes with a dominant-negative LMO4 molecule – lend support for the idea that LMO4 upregulation may contribute to the tumorigenic characteristics of mammary epithelial cells (Visvader *et al.*, 2001).

Acknowledgements
We thank Philip Leder for the MMTV plasmid; Ingolf Bach for Clim antisera; Jane Visvader for LMO4 antibody; Gordon Gill and Geof Rosenfeld for suggestions; and Taosheng Huang, Kristen Jepsen, and Steven M Lipkin for reading the manuscript. This work was supported by the NIH award AR02080, the Department of the Army award DAMD17-00-1-0182, the California Breast Cancer Research Program award 5JB-0119 (to BA.), and the Department of the Army award DAMD17-01-1-0183 (to NW).

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THE POTENTIAL ROLE OF A NEW LIM FACTOR, LMO4, IN BREAST CANCER

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Many properties of breast cancer cells, including increased proliferation and invasion, are common to epithelial cells of the developing mammary gland, suggesting that understanding of developmental control in normal mammary glands may provide important insights into the biology of breast cancer. This notion is supported by work in many organ systems, demonstrating that subversion of developmental control genes plays roles in carcinogenesis. LIM domain factors and associated co-regulators are important developmental regulators involved in pattern formation and organogenesis in a wide spectrum of organisms, including mammals. We isolated a LIM only factor, LMO-4, which is highly expressed in epithelial cells, including mammary epithelium. Interestingly, LMO factors are known to be oncogenic in lymphocytes where their overexpression causes acute lymphocytic leukemia.

We have studied expression of LMO-4 in mammary glands of mice and found that it is most highly expressed in proliferating mammary epithelial cells during pregnancy, suggesting that the LMO-4 gene may play a role in proliferation. Since LMOs do not bind to DNA it is likely that they regulate transcription by interacting with DNA-binding proteins and transcriptional co-regulators. To search for such factors, we have screened a human breast cDNA library with LMO-4 as bait in the yeast two hybrid system and found several potential interacting partners, including DNA-binding proteins, Clim/Nli/Ldb co-regulators and a splicing factor previously shown to be amplified in breast cancer cell lines. To test the role of LMO-4 in mammary gland biology, we have generated three lines of transgenic mice expressing under control of the MMTV promoter a) wild-type LMO-4, b) LMO-4 fused to the VP-16 transactivation domain and c) LMO-4 fused to the engrailed repression domain. Whole mount mammary gland analyses of these transgenic mice is in progress and preliminary results will be presented. Analyses of the EST databases indicate that LMO-4 is highly expressed in mammary carcinomas and we are in the process of evaluating its expression in breast cancer.

We conclude that LMO-4 may be an important regulator of mammary epithelial cells and propose a hypothesis that its high level expression in mammary tumors may play a role in mammary carcinogenesis.

The U.S. Army Medical Research and Materiel Command under DAMD17-00-1-0182 and DAMD17-01-1-0183 supported this work.

California Breast Cancer Research Program meeting in San Diego 2003.

Symposium Abstract (2003)

Many properties of breast cancer cells, including increased proliferation and invasion, are common to epithelial cells of the developing mammary gland. This suggests that understanding of developmental control in normal mammary glands may provide important insights into the biology of breast cancer. This notion is supported by work in many organ systems, demonstrating that subversion of developmental control genes plays role in carcinogenesis. LIM domain factors, and associated co-regulators, are important developmental regulators involved in pattern formation and organogenesis in a wide spectrum of organisms, including mammals. We isolated a LIM only factor, LMO-4, which is highly expressed in epithelium cells, including mammary epithelium. Interestingly, LMO factors are known to be oncogenic in lymphocytes where their overexpression causes acute lymphocytic leukemia.

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Heregulin/Her2 Regulation of LMO4 in Breast Cancer Cell.

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The growth factor receptor Her2 is overexpressed in about 40% of breast cancer cases, where it is thought to induce tumorigenicity and metastasis of breast cancer cells. The ligand heregulin (HRG) activates the Her2 receptor and its downstream signal transduction via induction of heterodimeric complexes of Her2 with Her3 or Her4. LIM domain factors and associated co-factors are important developmental regulators in pattern formation and organogenesis. The LMO (LIM-only) family consists of four members (designated LMO1-LMO4), each of which contains two tandem LIM domains. Overexpression of LMO1 and LMO2 leads to acute lymphocyte leukemia. The more recently discovered LMO factor LMO4 is highly expressed in proliferating epithelial cells and frequently overexpressed in breast cancer, suggesting that LMO4 may contribute to pathogenesis of breast cancer. The regulation of LMO4 is poorly understood. In this study, we explored the regulation of LMO4 mRNA expression in MCF-7 cells by HRG. We treated MCF-7 cells with HRG β1(20ng/ml) and measured the LMO4 transcript levels at different time points by RNase protection assays. LMO4 transcripts were up-regulated by HRG and this effect could be partially blocked by an Her2 antibody, suggesting a role for Her2 in HRG-mediated up-regulation of LMO4. The observation that LMO4 may be downstream of HRG/Her2 is consistent with the findings that mesenchymally-expressed HRG, like LMO4, is strikingly upregulated in mid-pregnancy. Furthermore, overexpression of a dominant negative LMO4 (engrailed LMO4) under the MMTV promoter inhibits ductular development in virgin mice and alveolar development in pregnant mice. In summary, our findings suggest the possibility that LMO4 may participate in HRG/ErbB signaling in the mouse mammary gland and breast cancer.

Supported by NIH award AR02080, the Department of the Army award DAMD17-00-1-0182, the California Breast Cancer Research Program award 5JB-0119 (to BA.), and the Department of the Army award DAMD17-01-1-0183 (to NW).